AD_____

CONTRACT NUMBER DAMD17-96-C-6118

TITLE: A Longitudinal Study of Bone Turnover, Menopause, Aging, and Ethnicity as Risk Factors for Osteoporosis

PRINCIPAL INVESTIGATOR: Sonja M. McKinlay, Ph.D.

CONTRACTING ORGANIZATION: New England Research Institute, Inc.
Watertown, MD 02172

REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Annual (25 Sep 97 - 24 Sep 98)

4. TITLE AND SUBTITLE
A Longitudinal Study of Bone Turnover, Menopause, Aging and Ethnicity as Risk Factors for Osteoporosis

5. FUNDING NUMBERS
DAMD17-96-C-6118

6. AUTHOR(S)
Sonja M. McKinlay, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New England Research Institutes, Inc.
Watertown, MD. 02172

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

This four-year study is a very cost-efficient and timely longitudinal study of bone turnover markers in mid-aged women as they experience the menopause transition. Building on the multisite Study of Women Across the Nation (SWAN), already funded by the National Institutes of Aging and Nursing Research at the National Institutes of Health, this study proposes to analyze already collected and stored specimens of serum to measure bone formation (using an immunoradiometric assay of osteocalcin) and stored urine specimens to measure bone resorption (using urinary N-telopeptide of type I collagen). These two measures will be combined with data from SWAN on bone density (spine, hip and total body), ovarian aging (endogenous sex hormones and menstrual bleeding), medications, medical history, social and psychological assessments, and life style factors (exercise, diet, smoking, body mass) to address four research aims. For each of the aims, specific hypotheses will be investigated using data collected at up to four annual visits as well as menstrual bleeding data collected continually from monthly calendars kept by the subjects. To date, major progress on the study includes finalization of data management, data collection forms, manuals of operations, and routine shipment of specimens.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Osteoporosis	34
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

10/23/98

PI - Signature Date

Table of Contents

I. Introduction

This four-year study is a very cost-efficient and timely longitudinal study of bone turnover markers in mid-aged women as they experience the menopause transition. Building on the multisite Study of Women Across the Nation (SWAN), already funded by the National Institutes of Aging and Nursing Research at the National Institutes of Health, this study proposes to analyze already collected and stored specimens of serum to measure bone formation (using an immunoradiometric assay of osteocalcin) and stored urine specimens to measure bone resorption (using urinary N-telopeptide of type I collagen). These two measures will be combined with data from SWAN on bone density (spine, hip and total body), ovarian aging (endogenous sex hormones and menstrual bleeding), medications, medical history, social and psychological assessments, and life style factors (exercise, diet, smoking, body mass) to address four research aims. For each of the aims, specific hypotheses will be investigated using data collected at up to four annual visits as well as menstrual bleeding data collected continually from monthly calendars kept by the subjects. The aims and hypotheses are as follows:

AIM I: To evaluate the relationships between markers of bone turnover (osteocalcin and Type I collagen N-telopeptides) and markers of ovarian aging (reproductive hormone levels, menstrual bleeding patterns, symptoms).
Hypotheses
I.1 Higher baseline levels of and/or higher rates of change in bone turnover markers will be associated with a higher probability of transition to peri- or post-menopausal status in the 2-year study period.
I.2 Higher baseline levels of and/or higher rates of change in bone turnover markers will be associated with a higher rate of self-reported peri-menopausal symptoms.
1.3 The association of bone turnover baseline levels and/or changes over time with the probability of transition to peri- or post-menopause will vary with chronological age (for example, a 50 year old woman may have higher baseline turnover levels and lower rates of turnover change, while a 45 year old woman may have low baseline turnover levels and higher rates of turnover change associated with the same transition probabilities).
AIM II: To determine if one-time (baseline) measures of bone turnover markers or changes over time in these measures are associated with the rate of bone loss over a similar time period.
Hypotheses
II.1 Elevated levels of baseline bone turnover markers will be associated with a greater bone loss in the subsequent two-year period.
II.2 An increase in levels of bone turnover markers (measured by at least two points in a two-year period) will be more strongly associated with greater bone loss over the same period than a single, baseline measure of bone turnover.
AIM III: To assess the degree to which potential lifestyle risk factors for osteoporosis (diet, cigarette smoking, exercise, weight) modify the relationships between bone turnover and ovarian aging (Aim I above) and between bone turnover and bone density (Aim II above).
Hypotheses
III.1 Compared with non-smokers, smokers will have higher baseline levels of bone turnover markers and stronger associations between bone turnover markers and ovarian aging, and between bone turnover markers and bone loss.
III.2 Women with diets rich in phytoestrogens will have lower levels of bone turnover markers, and weaker associations between bone turnover markers and ovarian aging, and between bone turnover markers and bone loss.
III.3 Body weight will be inversely associated with bone turnover levels.
III.4 Heavier women with low levels of physical activity will have weaker associations between bone turnover markers and ovarian aging, and between bone turnover markers and bone loss.
AIM IV: To determine whether the nature or magnitude of the relationships between bone turnover and ovarian aging markers (Aim I above) and bone density (Aim II above) vary according to racial or ethnic

grouping, and whether racial/ethnic differences in lifestyle factors account for any differences with respect to bone turnover markers.

Hypotheses

IV.1 Compared to Caucasians, African American women will have higher levels of bone turnover at baseline. Asian (and Mexican American) women will have baseline bone turnover levels between those for Caucasian and African American women.

IV.2 Racial/ethnic differences in lifestyle factors – such as higher smoking rates among Caucasians and African Americans, greater dietary phytoestrogens among Asians, higher weight among African Americans (and Mexican Americans) – will account for much of the hypothesized differences in baseline levels of bone turnover markers.

IV.3 There will be no detectable differences among different racial/ethnic groups with respect to change over time in bone turnover marker levels.

IV.4 The relationships between bone turnover markers and bone density, and between bone turnover markers and ovarian aging, will vary across racial/ethnic groups, but these differences will be explained in large part by racial/ethnic differences in lifestyle factors and in baseline levels of bone turnover markers.

II. Body

A. Study Objectives

This four-year project seeks to:

A. Measure osteocalcin (from serum) and Type I collagen N-telopeptides (from urine) using specimens collected annually at three time points from 2,250 women at five Field Sites across the U.S.; and
B. Combine these data with pertinent data being collected concurrently on the same women as part of the recently funded SWAN to address the Research Questions and Hypotheses as delineated in Section B above. The results of analyses will be appropriately presented and disseminated.

In order to accomplish the two technical objectives, the following key tasks and timelines were identified at the time of the application:

TECHNICAL OBJECTIVE A:		Measurement and QA/QC of Osteocalcin and Type I collagen N-Telopeptides
Task 1:	Months 1-2:	Finalization of data acquisition protocol
Task 2:	Months 1-2:	Finalization of data forms/electronic file formatting
Task 3:	Month 3:	Finalization of Manual of Operations
Task 4:	Months 2-3:	Design/Testing and implementation of DMS
Task 5:	Months 4-39:	Monthly shipments of specimens to the Central Laboratory
Task 6:	Months 4-38:	Monthly transfer of data results from the Central Laboratory to the Coordinating Center
Task 7:	Months 5-39:	On-going monitoring of Laboratory performance, including site visits
Task 8:	Months 6-42:	Assessment of the stability of stored specimens using pooled samples

TECHNICAL OBJECTIVE B:		Integration of bone turnovers with SWAN data, analyses and results dissemination
Task 9:	Months 15-40:	Integration go study and SWAN data into analytic data sets as baseline and follow-up annual data become available
Task 10:	Months 18-47:	Completion of all analyses.
Task 11:	Months 18-47:	Dissemination of results.

B. Study Progress

Overall, the study has progressed well. The recruitment of participants into the study, particularly the ethnic minorities, occurred at a slower pace and at a greater expense to the parent study than was originally anticipated. Therefore, timelines for the technical objectives have been adjusted to accommodate these unforeseen changes.

1. Participant Recruitment

Recruitment into the cohort study was completed in December, 1997. Baseline data collection on those participants recruited toward the end of the recruitment period were completed by March, 1998. All recruitment goals for the study were met. Therefore, there are a total of 2,150 participants at the five sites currently participating in the bone densitometry and bone marker study. In addition, the first annual follow-up is nearing completion (95 % complete) and is scheduled to be completed by December 31, 1998. The second annual follow-up is currently in the field and will be continuing until December 31, 1999.

2. Completion of Tasks

Progress on Technical Objectives A and B are detailed below:

TECHNICAL OBJECTIVE A:		Measurement and QA/QC of Osteocalcin and Type I collagen N-Telopeptides
Task 1:	Months 1-2:	Finalization of data acquisition protocol

Completion of this task was reported in the October 31, 1997 annual report

Task 2:	Months 1-2:	Finalization of data forms/electronic file formatting

Completion of this task was reported in the October 31, 1997 annual report

Task 3:	Month 3:	Finalization of Manual of Operations

Completion of this task was reported in the October 31, 1997 annual report

Task 4:	Months 2-3:	Design/Testing and implementation of DMS

Completion of this task was reported in the October 31, 1997 annual report

Task 5:	Months 4-39:	Monthly shipments of specimens to Central Laboratory

Shipments of specimens are occurring on a monthly basis. To date, all baseline samples have been shipped to the laboratory and all follow-up 01 and 02 samples are shipped monthly as specimens are received.

Task 6: Months 4-38: Monthly transfer of data results from Central Laboratory to the CC

The laboratory requires that enough baseline and follow-up samples have been received so that the analytes may be run "in-batch" using samples matched by subject id for baseline and follow-up 01 pairs. Once the analysis for a batch occurs, the data are transferred from the bone marker laboratory to the Coordinating Center on a monthly basis. This procedure is tested and fully operational. To date, results have been run and completed on 1500 matched pairs for the urine N-Teleopeptide and the laboratory is currently in the process of completing matched pairs for the serum osteocalcin and the remaining N-Teleopeptide pairs. The N-Teleopeptide data have been transferred to the coordinating center. The serum osteocaclin data and the remaining N-Teleopeptide data are scheduled to be transmitted to the coordinating center in early December, 1998.

Task 7: Months 5-39: On-going monitoring of Laboratory performance, including site visits

The bone marker laboratory currently maintains quality control procedures which include internal and external quality assurances. The detailed SOP for the assays being run for this project and MRL's external certifications are included in the appendix. Overall, the laboratory has maintained a high degree of quality in their laboratory assays and are fulfilling the required QC activities.

Task 8: Months 6-42: Assessment of the stability of stored specimens using pooled samples

The SWAN study's Laboratory Oversight Committee is charged with regular review of the laboratory's Standard Operating Procedures and QC data. The LOC has reviewed all SOPs and QC data for assays associated with this study and have found them to meet or exceed all standards.

Task 9: Months 15-40: Integration go study and SWAN data into analytic data sets as baseline and follow-up annual data become available

The SWAN baseline data are soon to be released for analyses by all SWAN investigators. Baseline bone densitometry data, along with the baseline bone marker data are scheduled to be released in December, 1998.

Task 10: Months 18-47: Completion of all analyses.

Analyses of the SWAN bone densitometry data and the bone marker data are underway. Preliminary results on bone density and bone turnover markers are presented below.

BONE DENSITY RESULTS

Baseline bone mineral density (BMD) measurements of the lumbar spine in the AP projection, proximal femur, and total body have been completed on 2,097 women at the five centers. Of these women, 1,057 (50%) were Caucasian, 649 (31%) were African-American, 130 (6%) were Chinese (results on the remaining 120 Chinese women are pending), and 261 (12%) were Japanese.

Women were excluded from the following data analyses if they reported glucocorticoid use for at least 6 months, use of anticonvulsants or depo Provera for at least 1 year, or if they reported a history of hyperthyroidism, hypercalcemia, chronic liver disease, anorexia nervosa, or bulimia. Mean ($\pm$ SD) baseline BMDs of the remaining 1,907 women are shown below (for all women, premenopausal women only, and early perimenopausal women only). (Note: there were no differences in the percentage of women who were excluded from any of the ethnic groups).

Table 1. BMD measures (mean + SE) according to pre and perimenopausal status

BMD SITE*#	All Women	Premenopausal	Early Perimenopausal
	n=1,907	n=1,028	n=879
AP spine	1.09 ± 0.14	1.09 ± 0.14	1.08 ± 0.14
Femoral neck	0.85 ± 0.14	0.85 ± 0.14	0.85 ± 0.13
Total hip	0.97 ± 0.15	0.97 ± 0.15	0.98 ± 0.14
Whole body	1.13 ± 0.10	1.13 ± 0.10	1.13 ± 0.10

*All values in gm/cm^2; #values not corrected for bone size

No significant differences in BMD were observed between pre- and early perimenopausal women at any anatomic site. Therefore, results from pre- and early perimenopausal women are combined in all subsequent analyses.

Figure 1. Mean Baseline Bone Density Levels by Ethnicity

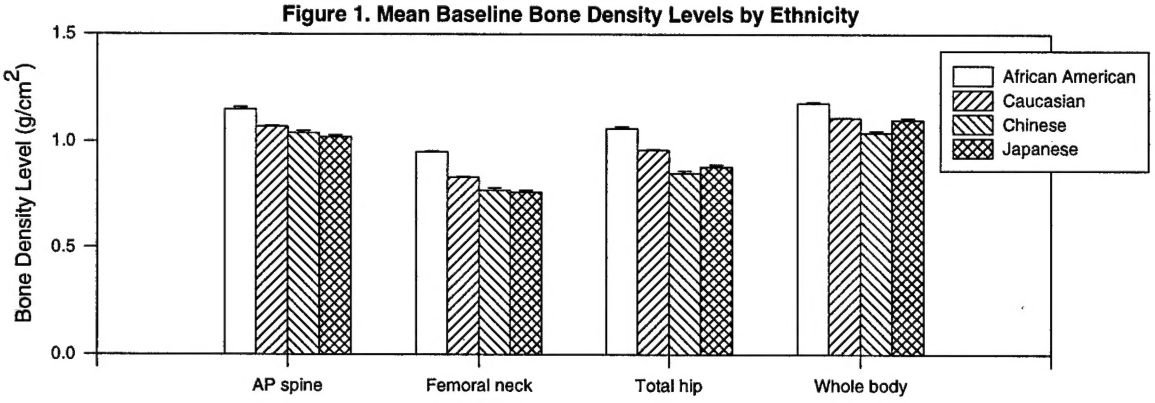


Table 2. BMD according to self-reported ethnicity in women (mean + SE)

BMD SITE*	African-American	Caucasian	Chinese	Japanese	p-value
AP spine	1.15 ± 0.01	1.07 ± 0.004	1.04 ± 0.01	1.02 ± 0.01	$p < 0.0001$
AP spine BMAD	1.25 ± 0.01	1.11 ± 0.01	1.06 ± 0.02	1.04 ± 0.01	$p < 0.0001$
Femoral neck	0.95 ± 0.005	0.83 ± 0.004	0.77 ± 0.01	0.76 ± 0.01	$p < 0.0001$
Total hip	1.06 ± 0.01	0.96 ± 0.004	0.85 ± 0.01	0.88 ± 0.01	$p < 0.0001$
Whole body	1.18 ± 0.004	1.11 ± 0.003	1.04 ± 0.01	1.10 ± 0.01	$p < 0.0001$

*All values in gm/cm^2

There were large differences in baseline BMD among ethnic groups at each anatomic site ($p<0.0001$ by ANOVA at each anatomic site). Baseline BMD at each anatomic site was significantly ($p<0.0001$) higher in African-American women than in women of the other 3 ethnic groups. Baseline BMD was similar in Chinese and Japanese women at each anatomic site except for whole body BMD (which was higher in Japanese women). Baseline BMD of the AP spine ($p=0.0105$ vs Chinese and $p<0.0001$ vs Japanese), femoral neck ($p<0.0001$), and total hip ($p<0.0001$) were all significantly higher in Caucasian women than in Chinese or Japanese women. Baseline whole body BMD was significantly ($p<0.0001$) higher in Caucasian than in Chinese women. Adjustment for thickness of the vertebral bodies by taking each BMD value to the 3/2 power, (BMAD) (123) did not alter any of these results. The results were virtually identical when BMD among ethnic groups was assessed separately in pre and perimenopausal women.

Table 3. Least squares mean of baseline BMD by ethnicity (mean + SE), adjusting for BMI and site

BMD SITE*	African-American	Caucasian	Chinese	Japanese	p-value for model
AP spine	1.13 ± 0.01	1.07 ± 0.004	1.06 ± 0.01	1.07 ± 0.01	0.0001
AP spine BMAD	1.20 ± 0.01	1.11 ± 0.01	1.10 ± 0.02	1.12 ± 0.02	0.0001
Femoral neck	0.91 ± 0.01	0.83 ± 0.004	0.81 ± 0.01	0.82 ± 0.01	0.0001
Total hip	1.01 ± 0.01	0.96 ± 0.004	0.94 ± 0.01	0.94 ± 0.01	0.0001
Whole body	1.17 ± 0.005	1.11 ± 0.003	1.09 ± 0.01	1.11 ± 0.01	0.0001

*All values in gm/cm^2

After adjusting for BMI and SWAN site, baseline BMD at each anatomic site was still significantly ($p<0.0001$) higher in African-American women than in women of the other 3 ethnic groups. There were, however, no pairwise differences among the other 3 ethnic groups (Caucasians, Chinese, and Japanese women) after adjustment for BMI and SWAN site. The differences in AP spine, femoral neck and total hip BMD between African-American and Caucasian women were reduced by 24%, 30%, and 48%, respectively, after adjusting for BMI and SWAN site.

A multiple variable regression model that included ethnicity, menopausal status (pre or peri), smoking (number of pack years), years of OCP (oral contraceptive pill) use, number of pregnancies, age, daily calcium intake, and BMI confirmed that ethnicity remained a statistically significant predictor of BMD, even when controlling for these other factors. These variables explained between 22% and 44% of the variability in BMD at each skeletal site. BMI was a major correlate of BMD at each site. Results were similar when lean body mass (by DXA) was substituted for BMI. Adjustment for thickness of the vertebral bodies (BMAD) did not alter any the results of the multiple variable models.

Table 4. Multivariable regression models for BMD [normalized β Coefficient (SE)].

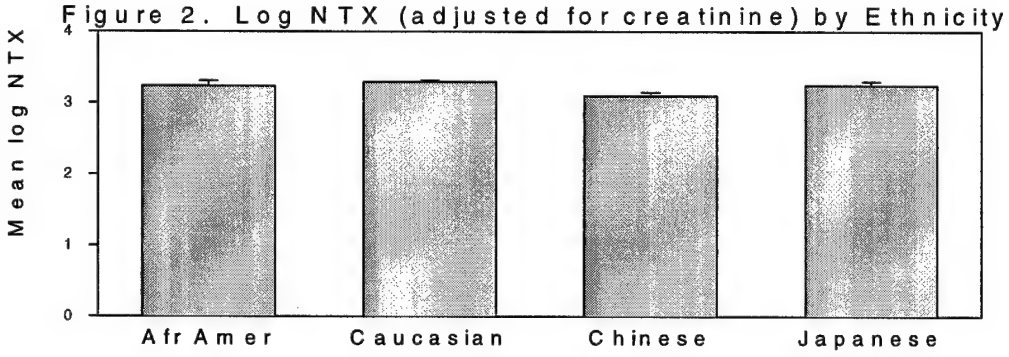
Predictor	AP Spine BMD	Femoral neck BMD	Total Hip BMD	Total Body BMD
Ethnicity				
African American	0.07 (0.01)****	0.09 (0.01)****	0.06 (0.007)****	0.06 (0.01)****
Chinese	-0.004 (0.01)	-0.02 (0.01)	-0.06 (0.01)****	-0.06 (0.01)****
Japanese	-0.01 (0.01)	-0.02 (0.01)*	-0.02 (0.01)*	0.01 (0.01)
Caucasian (ref)				
Menopause status				
Pre (ref) Early peri	-0.01 (0.01)	-0.01 (0.01)*	-0.01 (0.01)	-0.01 (0.004)
Smoking (pack-yrs)¶	0.009 (0.08)	0.05 (0.06)	0.06 (0.07)	-0.04 (0.05)
OCP use (# yrs)	0.0002 (0.001)	0.0003 (0.001)	-0.0002 (0.001)	-0.001 (0.0005)
Pregnancies (#)	-0.002 (0.002)	0.001 (0.001)	0.001 (0.001)	0.001 (0.001)
Age (yrs)	-0.0004 (0.001)	-0.002 (0.001)*	-0.001 (0.001)	-0.001 (0.001)
Ca^{++} intake (mg/d)¶	-0.25 (0.56)	0.55 (0.46)	0.23 (0.50)	-0.28 (0.78)
BMI (kg/m^2)	0.01 (0.0005)****	0.01 (0.0004)****	0.01 (0.0004)****	0.01 (0.001)****
R^2	22%	43%	44%	22%
N	1619	1641	1641	1612

*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
¶coefficient and SE have been multiplied by 100,000

The results were similar when the aforementioned models were run separately in strata of only pre- or perimenopausal women. In this analysis, BMD of the total hip and total body was lower in Chinese than in Caucasian women. BMD of the femoral neck and total hip was lower in Japanese than in Caucasian women.

BONE TURNOVER MARKER RESULTS

Baseline urinary NTX/creatinine excretion was significantly lower in Chinese women than in Caucasian ($P<0.001$) or Japanese ($p<0.05$) women. Baseline urinary NTX excretion was similar among African-American, Caucasian, and Japanese women (see Figure 2). When considering only the premenopausal women, NTX was significantly lower in African-American women than in Caucasians ($p=0.033$). When considering only the perimenopausal women, NTX was higher in African-American than in Chinese women ($p=0.004$), and lower in Chinese women than in Caucasians ($p=0.001$). In a multiple regression model that included ethnicity (Caucasians are the reference group), menopausal status, smoking, prior OCP use, number of pregnancies, age, calcium intake and BMI (or lean mass), Chinese and Japanese women had significantly lower NTX levels than Caucasian women. Total dietary calcium and BMI were negatively associated with NTX levels. However, the R^2 for the model was only 5.4%.



RELATING BONE DENSITY TO BONE TURNOVER MARKERS

The natural logarithm of baseline NTX/creatinine was significantly negatively correlated with spine ($p=0.0001$), hip ($p=0.0001$), and whole body ($p=0.0001$) BMD, although the R^2 values were low (ranging from 0.005 to 0.04). NTX was not significantly correlated with lateral BMD (see Figures 3-6).

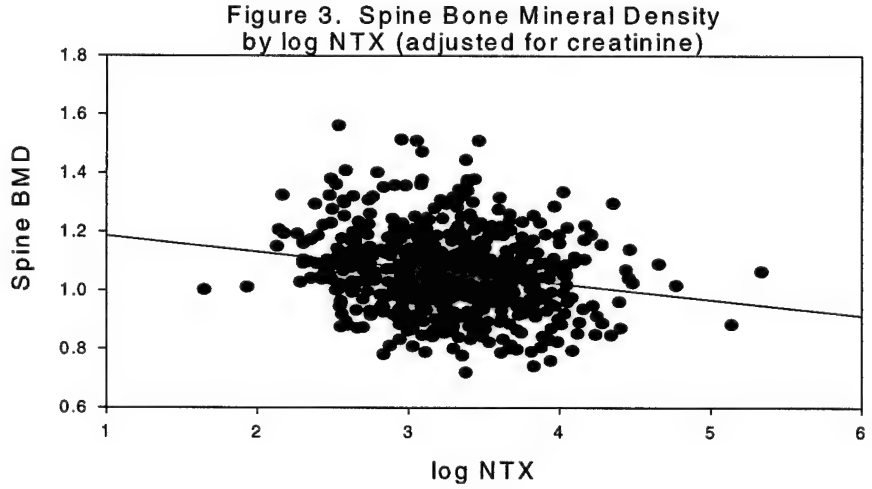


Figure 4. Hip Bone Mineral Density
by log NTX (adjusted for creatinine)

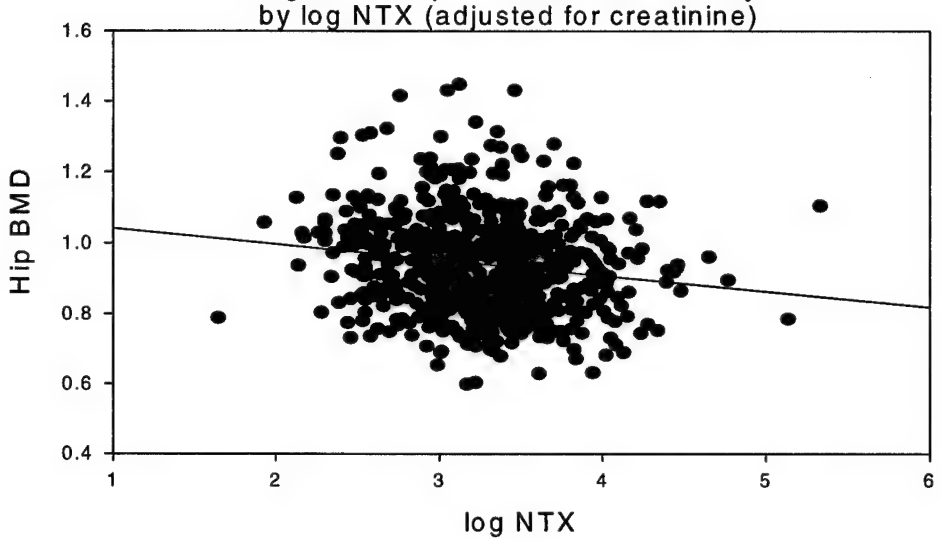
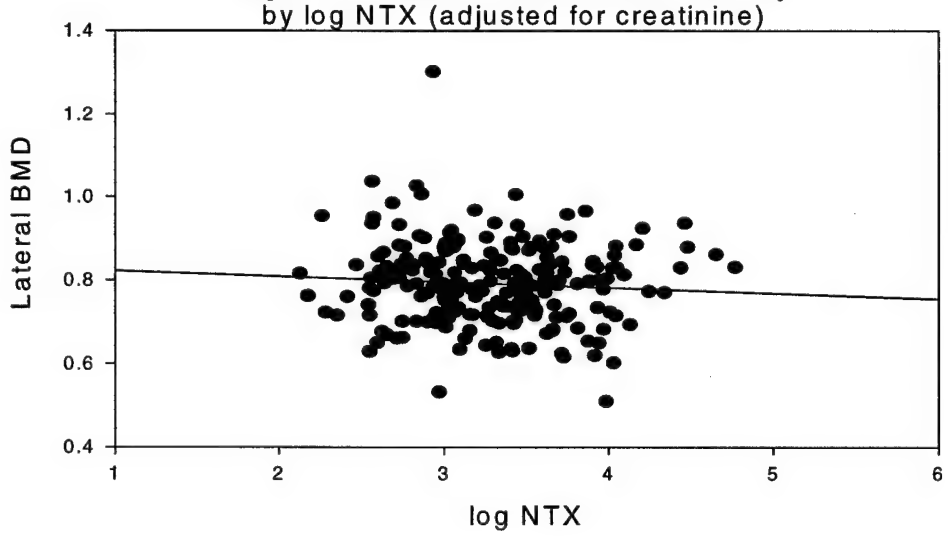
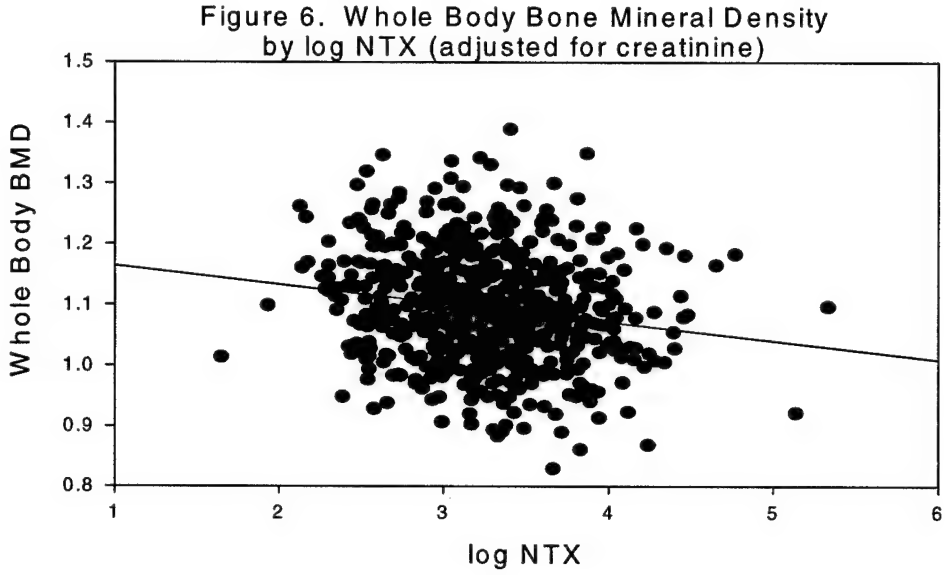


Figure 5. Lateral Bone Mineral Density
by log NTX (adjusted for creatinine)





Task 11: Months 18-47: Dissemination of results.

Writing groups are now gearing up to write manuscripts that will disseminate the results of the baseline data collection. We anticipate manuscript development to continue throughout the study period and expect the first manuscripts to be submitted by the middle of 1999.

Conclusions

We are well on the way to completing the objectives of this project. During the next year, we anticipate having longitudinal data available to report change in bone density in relation to change in bone turnover markers. We also anticipate that during this year, the first manuscripts reporting baseline data will be submitted to professional journals and will be presented at professional meetings.

References

Not Applicable.

Appendix

Medical Research Laboratory SOP for Urine N-Telopeptide, Urine Creatinine, and Serum Osteocalcin and external QC certifications

MRL
Medical Research Laboratories

Standard Operating Procedure
Procedure LAB #6000.1
Department: Laboratory
Lab Area: Miscellaneous

TITLE: N-TELOPEPTIDE OF TYPE I COLLAGEN IN HUMAN URINE BY ELISA

Date issued: 16. January 1996
Written or Revised by: Judy Miller Manager, QA, R&D
7 Pages
Replaces Procedure: **SOP LAB #6000 (28. October 1994)**
Approved by: Evan Stein President

Purpose: To provide a standardized procedure, using NCCLS guidelines, for performing the N-telopeptide of Type I collagen assay in the laboratory.

Contents:

Definitions:

Valid for: All MRL personnel

References:

1. Osteomark™ Assay Package Insert by Ostex International, Inc., 2203 Airport Way South, Suite 301, Seattle, WA 98134, 1993.

2. Jeff Morgan, Project Leader, Product Development for Ostex International, Inc., 3-2-93.

3. Daniel D. Bickford, Development Associate, Ostex International, Inc., 2-2-94.

4. Hanson DA, Weis MAE, Bollen AM, Maslan SL, Singer FR, and Eyre DR. "A Specific Immunoassay for Monitoring Human Bone Resorption: Quantitation of Type I Collagen Cross-linked N-Telopeptides in Urine". Journal of Bone and Mineral Research 7(11), 1992.

Procedure:

1. Principle

1.1. Osteomark™ is an enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of the N-telopeptide cross-linking domain of bone collagen in human urine. The

solid phase consists of microwells onto which antigen is adsorbed. Urine controls and test samples are diluted in Specimen Diluent and added to coated Microwells, along with Calibrators provided in the kit. Antibody-Horseradish Peroxidase Conjugate is then added to each well. During an initial incubation period, antigen in the sample competes with the solid-phase antigen for binding to the antibody. The wells are then washed to remove unbound material.

1.2. Buffered Substrate/Chromogen Reagent (hydrogen peroxide and 3,3',5,5' tetra-methylbenzidine) is then added to each well. During the final incubation, a blue color will develop in a reaction when bound Antibody-Horseradish Peroxidase Conjugate is present in the well. The amount of color that develops is a measure of the amount of conjugated antibody bound to the antigen adsorbed to the solid phase, and is indirectly proportional to the amount of antigen in the test sample. The reaction is stopped by addition of Stopping Reagent (1N sulfuric acid) which results in a color change from blue to yellow. The absorbance values for the Controls, Calibrators, and test samples are determined spectro-photometrically at 450 nm using 650 nm reference, by using a microplate reader.

1.3. N-telopeptide of Type I collagen in urine is used for monitoring bone resorption rates in humans. Since the cross-linked fragment of Type I collagen is excreted as a reproducible fraction of total bone-derived pyridinoline, it can act as a quantitative measure of the systemic role of bone resorption.

2. Specimen:

2.1. The determination is performed on either a 24-hour urine collection or a single "midstream" urine collection. The urine must be preservative free. Store refrigerated (2-8°C) for up to 24 hours. Store frozen (-20°C or below) for longer term storage.

2.1.1. "Midstream" urine collection: Patients should be instructed to collect a midstream sample, discarding approximately the first one-half of the urine voided, collecting the sample, and allowing the remainder to pass into the toilet.

2.2. Allow no more than 5 freeze/thaw cycles of the specimen.

2.3. Frozen specimens which are being assayed should be warmed to 37°C in a waterbath for 20 minutes and rotated to mix for 10 minutes.

3. Reagents:

3.1. All reagents are supplied in the Osteomark™ Assay Kit by Ostex (Catalog #PN9006).

3.1.1. Antigen coated 96-well plate (each plate contains 12 removable strips)

3.1.2. Antibody Conjugate Diluent

3.1.3. Antibody Conjugate Concentrate

3.2. A working conjugate solution is prepared by diluting the Antibody Conjugate Concentrate 1:101 in Antibody Conjugate Diluent. Prepare approximately 2 mL of working conjugate for each strip tested. If an entire plate is tested, dilute 240 μL of Antibody Conjugate Concentrate into 24 mL of Antibody Conjugate Diluent. Use within one hour of preparation.

3.3. Calibrators of 1, 30, 100, 300, 1000, and 3000 pMoles BCE/mL. Ready to use.

3.4. Buffered Substrate

3.5. Chromogen Reagent

3.5.1. A Chromogen/Buffered Substrate is prepared by making a 1:101 dilution of Chromogen Reagent into buffered substrate using the volumes in the table below.

Total Number of Strips	Chromogen Reagent (µL)	Buffered Substrate (mL)
2- 4	80	8
5- 8	160	16
9-12	240	24

3.5.2. Invert gently to mix. **DO NOT VORTEX.**

3.6. 30X Wash Concentrate
3.6.1. Dilute 1:30 in deionized water. Mix well.

3.7. Stopping Reagent
3.7.1. 1N sulfuric acid. Ready to use.

3.8. Control Levels I and II
3.8.1. Ready to use.

3.9. Plate Sealers
3.9.1. A cellophane cover for incubation.

3.10. Reagent Storage and Stability:
3.10.1. Storage conditions: Store all kit reagents, except diluted wash solution, at 2-8°C. Diluted wash solution may be stored at room temperature for up to one month. Return all reagents to the storage conditions indicated.

4. Supplies
4.1. COSTAR Cluster Tubes. Catalog Number 4410.
4.1.1. 2 mL polypropylene tubes arranged in the same format as the microtitre plate.
4.2. COSTAR Pipette Tips. Catalog Number 4865.
4.2.1. Long pipette tips to facilitate pipetting out of the cluster tubes.
4.3. COSTAR Cluster Tube 8-Cap Strip. Catalog Number 4418.
4.3.1. Caps for cluster tubes after use.
4.4. 8-Channel Multipipettor, 5-50 µL range.
4.5. 8-Channel Multipipettor, 50-200 µL range.

5. Equipment
5.1. Vortex mixer
5.2. Multi-Channel Plate Washer
5.3. Behring ELISA Processor

6. Quality Control
6.1. Three levels of osteomark QC pools made from donor urine are included with each run.
6.2. Control values must have a coefficient of variation of less than 15%.
6.3. If any level of QC is outside of established limits, the entire run is repeated. QC limits are as follows:
6.3.1. 1_{3S}: Reject run if 1 of 3 levels of QC is greater than 3 SD from the mean.
6.3.2. $(2 \text{ of } 3)_{2S}$: Reject run if 2 of 3 levels of QC is greater than 2 SD from the mean.
6.3.3. R_{4S}: Reject run if 2 of 3 levels of QC exceed opposite ends of mean by 2 SD (e.g., 1=> + 2 SD, 1 = > -2 SD). Quality control is plotted daily on a Levy-Jennings graph.

7. Procedure:
7.1. Remove all reagents from the refrigerator. The Chromogen Reagent contains dimethylsulfoxide (DMSO) and will solidify upon refrigerated storage. ALLOW CHROMOGEN REAGENT

AND BUFFERED SUBSTRATE TO COME TO ROOM TEMPERATURE BEFORE BEGINNING STEP 2 INCUBATION. Thaw frozen specimens at room temperature. Gently vortex Calibrators, Controls, and specimens. Avoid foaming.

7.2. Aliquot standards, controls, and patients into appropriately labeled cluster tubes.

7.3. Remove appropriate number of microtitre strips from the sealed pouch. Reseal along the zipper.

7.4. Write sample identification on a worksheet.

7.5. Prepare working conjugate solution using instructions listed under REAGENTS.

7.6. Pipet 25 µL of standards, controls, and patients in duplicate into appropriate wells. This is best performed one row at a time using an 8-channel multipipettor and appropriately aligned cluster tubes. Use diagram in Figure 1.

FIGURE 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1										
B	30	30										
C	100	100										
D	300	300										
E	1000	1000										
F	3000	3000										
G	I	I										
H	II	II										

7.7. Gently mix the Working Conjugate Solution. Using an 8-channel multipipettor, pipet 200 µL of Working Conjugate Solution into each well. Pipet into all wells as quickly as possible to minimize the difference in time between the first wells and the last wells. Swirl plate gently to mix and cover with a plate sealer.

7.8. Incubate at room temperature (18-25°C) for 90 ± 15 minutes.

7.9. During incubation, prepare Wash Solution using instructions listed under REAGENTS.

7.10. Ten (10) minutes prior to the completion of the incubation, prepare the Chromogen/Buffered Substrate using instructions listed under REAGENTS.

7.11. At the end of the first incubation, carefully remove (pulling diagonally) and discard the Plate Sealer and aspirate the well contents. Wash FIVE (5) times with diluted Wash Solution as described under Procedural Notes. Make sure that undersides of the strips are dry. If not, wipe them gently with a tissue. Aspirate residual fluid from the wells and blot dry. Immediately add Chromogen/Buffered Substrate as described in Step 12.

7.12. Pipet 200 µL of Chromogen/Buffered Substrate into each well using an 8-channel multipipettor. Cover with a new plate sealer.

7.13. Incubate at room temperature for 15 ± 1 minute. A blue color will develop in wells containing bound Antibody-HRP Conjugate.

7.14. At the end of the second incubation, carefully remove and discard the Plate Sealer. Add 100 uL of Stopping Reagent to each well using an 8-channel multipipettor. The wells which have developed a blue color will turn yellow.

7.15. Read absorbance on a microplate or microstrip reader at 450 nm with a reference filter for

reading at 650 nm within 30 minutes of adding Stopping Reagent.

7.16. Quantitative software with a 4-parameter curve fitting equation must be used to analyze the data.

8. Calculations:

8.1. Determine concentration values (picomole Bone Collagen Equivalents/mL) of specimens and controls from the calibration curve. Accurate results are best obtained using a 4-parameter curve fitting equation.

8.2. Test specimens from single urine collections may be normalized for urine dilution by urinary creatinine measurement. Final results are reported as picomoles BCE/umole creatinine.

$$\frac{pM\ BCE/mL}{creatinine\ mg/dL} \times 11.3 = pM\ BCE/uM\ creatinine$$

9. Reporting Results:

9.1. Results are reported in whole numbers using pM BCE/uM creatinine as units.

9.2. Patient values must have a CV of less than 20%. If the CV is greater than 20%, the sample must be repeated.

9.3. The mean absorbance value of the 1 pM BCE/mL calibrator must be greater than 1.500.

9.5. The span of the calibrator curve (absorbance difference between 1 pM BCE/mL and 3000 pM BCE/mL calibrators) should be ≥ 1.300 absorbance value.

10. Reference Range:

10.1 All units are pM BCE/uM creatinine.

STUDY GROUPS	MEAN	STANDARD DEVIATION	RANGE	n
Children, years 0-1	1639	915	102-4769	125
2-5	689	294	34-1752	269
6-10	497	667	90-1356	321
11-15	429	215	34-2158	239
16-20	192	147	34-780	83
Premenopausal females (mean age 30 years; range 25-40 years)	36	10	10-89	32
Postmenopausal females (< 3 years after menopause; mean age 51 years; range 42-58 years)	82	33	28-194	56
Males (mean age 30 years; range 24-40 years)	39	22	14-87	18

Males (mean age 55 years; range 45-65 years)	38	13	14-59	14

11. Procedural Notes:

11.1. Pipetting: Calibrators, Controls, and specimens should be pipetted into the bottom of the wells. Change disposable pipette tip after each specimen transfer to avoid cross-contamination.

11.2. Washing procedure: It is important to wash the microwell strips five times with diluted wash solution. Too few or too many washes may give less reproducible or inaccurate results. The strips may be washed with either a strip washer or a squirt bottle. Do not allow wells to dry out between reagent additions.

11.3. Automated washer: Follow the manufacturer's instructions. First aspirate the fluid from each well. For each wash, the wells should be filled to 400 uL with diluted wash solution. The contents should then be aspirated. Repeat until 5 washes have been performed. Blot the microwell plate on a pad of clean paper towels to remove excess wash solution.

11.4. Squirt bottle: First aspirate the fluid from each well. For each wash, grasp the long edges of the strip holder from beneath with thumb and opposing fingers while applying slight pressure and hold it horizontally over a sink. Fill the wells with diluted wash solution from a squirt bottle. In one movement, invert the strip holder and empty the wells into the sink with a flick of the wrist, holding the strip holder tightly to keep the strips in place. Repeat until 5 washes have been performed. While the plate frame is still inverted, flick it again and then blot it on a pad of clean paper towels.

11.5. Working strength conjugate should be used within 1 hour of preparation. Always use a clean disposable container for preparation of this reagent. NEVER REUSE THE CONTAINER.

11.6. Chromogen/Buffered Substrate: This should be colorless when mixed, prior to use. A blue color indicates that the reagent has been contaminated and should be discarded.

11.7. The Antigen Coated 96-well Plate, Calibrators and Controls of this kit contain components of human urine and human bone tissue. These components should, therefore, be handled as potentially biohazardous material.

11.8. Handle Chromogen Reagent with care, since it contains dimethylsulfoxide (DMSO), which is readily absorbed through the skin.

11.9. The Stopping Reagent is a strong acid. Wipe up spills immediately. Flush the area of the spill with water. If Stopping Reagent contacts the skin or eyes, flush thoroughly with water and seek medical attention.

12. Limitations of Procedure

12.1. This assay is sensitive down to 10 pM BCE/mL. Any patient value less than 10 pM BCE/mL should be reported as below detectable limits.

12.2. This assay is linear up to 3,000 pM BCE/mL. If the specimen exhibits a value of greater than 3,000 pM BCE/mL, dilute the specimen appropriately with deionized water and retest.

SIGNATURES

Procedure LAB #6000

Originator(s): Judy Miller Date: 1-18-96

_____ Date: _____

_____ Date: _____

_____ Date: _____

Approved By: (must be signed by either the Head of the Department, the President, Vice President of Operations, or Manager of Quality Assurance and R&D.)

Approval: _____ Date: 1/22/96

Title: PRESIDENT

Yearly Review:

Reviewed By: Judy Miller Date: 1/97

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

MRL
Medical Research Laboratories

Standard Operating Procedure
Procedure LAB #4224
Department: Laboratory
Lab Area: Chemistry

TITLE: CREATININE IN SERUM, PLASMA OR URINE - HITACHI 747

Date issued:	22. March 1995	
Written or Revised by:	Traci Turner	Assistant Laboratory Manager
	Kathy Dick	Senior Medical Technologist
6 Pages		
Replaces Procedure:	*TT/dzb/oct11c* (1993)	
Approved by:	Evan Stein	President

Purpose: To provide a standardized procedure, using NCCLS guidelines, for performing the creatinine assay on the Hitachi 747.

Contents:

Definitions: NCCLS
National Committee for Clinical Laboratory Standards

Valid for: All MRL personnel

References:

1. Jaffe M: Ztschr Physiol Chem, 10:391, 1886.
2. Henry RJ: Clinical Chemistry Principles and Technics, Harper & Row, New York, 1969, p. 287.
3. Teger-Nilsson AC: Scand J Clin Lab Invest, 13:326, 1961.
4. Creatinine Application Sheet, Boehringer Mannheim Diagnostics, Indianapolis, IN, 1990.
5. Kaplan LA, Pesce AJ: Methods in Clinical Chemistry, C.V. Mosby Co., pp. 16, 1297-8, 1987.
6. Jacobs, David S. Laboratory Test Handbook. Lexi-Comp/Mosby, 1988, p. 101.

Procedure:

1. Principle:

1.1. In alkaline medium, creatinine forms a yellow-orange-colored complex with picric acid.

$$\text{creatinine} + \text{picric acid} \xrightarrow{\text{alkaline}} \text{creatinine-picric acid complex}$$

1.2. The color intensity is proportional to the concentration of creatinine present and is measured photometrically at 505 nm.

1.3. Creatinine measurements are used as a renal function test, providing an index of glomerular filtration.

2. **Specimen:**

2.1. Use serum (SST or plain tubes) or plasma, free of icteria. Heparin or EDTA in the usual concentrations will not interfere. Use of other common anticoagulants will interfere with the test.

2.1.1. No special patient instructions are required.

2.1.2. Creatinine is stable in serum or plasma for 24 hours at 2-8°C. For longer storage, freeze specimens at -20°C.

2.2. For a random urine, patients should be instructed to collect a midstream sample, discarding approximately the first one-half of the urine voided, collecting the sample, and allowing the remainder to pass into the toilet.

2.3. For creatinine clearance determination, one needs a precisely timed urine collection and a blood sample taken during the collection period. A 24-hour collection yields the best results.

2.3.1. To initiate the test, the patient empties his/her bladder at the beginning of the timed period. Urine is collected throughout the period, and the bladder is again emptied at the end of the timed period.

2.3.2. No special preservative is necessary for the urine collection, though the urine should be refrigerated at 4-8°C.

2.3.3. The 24-hour urine volume is measured and a 10 ml aliquot is centrifuged at 2400 g for 10 minutes. Dilute urine x 10 with 0.9% NaCl prior to analysis.

3. **Reagents:**

3.1. Reagents are obtained from Boehringer Mannheim Corporation.

3.2. **NaOH** (Catalog #1127632) (5 x 600 mL)

3.2.1. Reactive Ingredient:
0.2 mol/L Sodium hydroxide

3.3. **Picric Acid** (Catalog #1127659) (5 x 300 mL)

3.3.1. Reactive Ingredient:
25 mmol/L Picric Acid

3.4. **Precautions and Warnings:**

3.4.1. For in vitro diagnostic use.

3.4.2. Never pipette by mouth. Exercise the normal precautions required for the handling of all laboratory reagents.

3.4.3. **WARNING. CORROSIVE.** Bottle 1 contains sodium hydroxide. In case of contact, flush affected areas with copious amounts of water. Get immediate medical attention for eyes or if ingested.

3.4.4. **DANGER. TOXIC.** Bottle 2 contains picric acid. In case of contact, flush affected areas with copious amounts of water. Get immediate medical attention for eyes or if ingested.

3.5. Reagent Preparation:

3.5.1. Preparation of Working Solutions:

3.5.1.1. For R1 Working Solution, use contents of one Bottle 1 (NaOH) as supplied. No preparation is required.

3.5.1.2. For R2 Working Solution, use contents of one Bottle 2 (Picric Acid) as supplied. No preparation is required.

3.5.2. Storage and Stability:

3.5.2.1. Store system reagents at 20-25°C. For stability of the unopened components, refer to the box or bottle labels for the expiration dates.

3.5.2.2. The opened R1 Working Solution is stable at 2-12°C for three weeks.

3.5.2.3. The opened R2 Working Solution is stable at 2-12°C until the expiration date on the bottle.

4. **Calibration:**

4.1. See Standard Operating Procedure LAB #4202 for instructions regarding calibration.

5. **Quality Control:**

5.1. Unassayed normal and abnormal pools are obtained from Ciba-Corning (Product Code 971600/971700). See Standard Operating Procedure LAB #4502 for guidelines to evaluate unassayed control pools. See Standard Operating Procedure LAB #4202 for instructions for preparing and running quality control pools as well as for acceptance of data.

5.2. Instrumentation Laboratories Urichem Control for urine (Catalog #2934-80). Reconstitute vial volumetrically with 25 ml XXH_2O. Aliquot and freeze at -70°C. Stable one year at -70°C. Dilute x 10 with 0.9% NaCl prior to analysis.

5.3. Normal serum pool - pool fresh serum (value ≈ 1.0), aliquot, and freeze at -70°C. Stable one year at -70°C. Run once daily.

6. **Instrument Settings:**

6.1. **4.2. Chemistry Parameters:**

Temperature: 37°C

TEST NAME	:[CREA]
ASSAY CODE	:[RATE-A] [30]-[35]
WAVELENGTH (SUB)	:[570] nm

WAVELENGTH (MAIN)	:[505] nm	
	SERUM	URINE
SAMPLE VOLUME	:[10] [10]	[10] [10]
EXPECTED VALUE	:[0.7]-[1.4]	[0]-[150]
PANIC VALUE	:[0.4]-[1.9]	[0]-[150]
ABS LIMIT	:[4500] [INCREASE]	[4500] [INCREASE]
PROZONE LIMIT	:[0] [LOWER]	[0] [LOWER]
R1 VOLUME	:[250] uL	
R2 VOLUME	:[50] uL	
R1 DUMMY INTERVAL	:[0]	
R2 DUMMY INTERVAL	:[0]	
DILUTION VOLUME	:[0]	

CALIBRATION METHOD	:[LINEAR]	STD	CONC	RACK	POS
POINTS	:[0]	1(SALINE)	0	1	1
SD LIMIT	:[0.1]	2(PRECICAL)	*	1	2
DUPLICATE LIMIT	:[10]	3	0	0	0
SENSITIVITY LIMIT	:[0]	4	0	0	0
STD1 ABS LEVEL	:[0]-[4000]	5	0	0	0
INSTRUMENT FACTOR	:[1.0]	6	0	0	0

*ASSIGNED VALUE

7. **Calculation:**

7.1. The analyzer computer uses absorbance measurements to calculate creatinine concentration as follows:

$$Cx = K(Ax - Ab) + Cb$$

Where:

Cx = Concentration of Sample
K = Factor for determining creatinine concentration, determined by the use of the calibrator
Ax = Change in absorbance per minute of Sample + R1 + R2 from positions 30 through 35
Ab = Change in absorbance per minute of Blank + R1 + R2 from positions 30 through 35
Cb = Concentration of Reagent Blank

7.2. To calculate clearance, use the following formula:

$$\text{Clearance} = \frac{UV}{P} \times \frac{1.73}{S} = \frac{\text{urine creatinine(mg/dl)}}{\text{serum creatinine(mg/dl)}} \times \frac{\text{volume(ml)}}{\text{time(min)}} \times \frac{1.73}{S}$$

Where:

S = Calculated surface area of the patient
1.73 = surface area (m^2) of a standard 70 kg person

See attached nomogram for correcting body weight and height to surface area.

8. **Reporting Results:**

8.1. Results are reported to one decimal point in mg/dl.
8.2. Reference Range:
8.2.1. Serum: 0.7 - 1.4 mg/dl
8.2.2. 24-hour Urine: 1.0 - 1.8 gm/TV
8.2.3. Creatinine Clearance:
8.2.3.1. Male: 75 - 135 ml/min
8.2.3.2. Female: 65 - 125 ml/min

9. **Procedural Notes:**

9.1. The "Compensated Test" display of the PARAMETER job is used to multiply the urine result by 10. On the "Compensated Test Entry": CREAT. TEST # (URINE) = CREAT X 10.

10. **Limitations of Procedure:**

10.1. No significant interference from hemoglobin (686 mg/dl) or lipemia (1,120 mg/dl triglycerides) is observed.
10.2. Significant negative interference from bilirubin at levels > 10 mg/dl is noted.
10.3. For ketones, acetone to 50 mg/dl, acetoacetate to 20 mmol/L, and β-hydroxybutyrate to 25 mmol/L do not significantly interfere with the assay.
10.4. The assay is linear up to 15.0 mg/dl.
10.5 The assay is sensitive to 0.1 mg/dl.

SIGNATURES

Procedure LAB #4224

Originator(s): Terri Turner Date: 4-11-95

Kathy L Dick Date: 4-11-95

Date: _____

Date: _____

Approved By: (must be signed by either the Head of the Department, the President, Vice President of Operations, or Manager of Quality Assurance and R&D.)

Approval: _____ Date: 4/11/95

Title: PRESIDENT

Yearly Review:

Reviewed By: Judy Miller Date: 4/12/96

Reviewed By: Judy Miller Date: 7/16/97

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

TITLE: OSTEOCALCIN IN SERUM OR PLASMA BY RIA

Date issued:	15. March 1997	
Written or Revised by:	Jerome C. Becker	Senior Medical Technologist
5 Pages		
Replaces Procedure:	First Issue	
Approved by:	Evan Stein	President

Purpose: To provide a standardized procedure, using NCCLS guidelines, for performing osteocalcin in serum or plasma by RIA.

Contents:

Valid for: Special Chemistry personnel.

References:
1. Directional Insert, ELSA-OSTEO, CisBio International, France, 1995.

Procedure:

1. Principle:
   1.1. Osteocalcin or Bone GLA Protein (BGP) is a low molecular weight protein. Synthesized by osteoblasts, it is specific to bone tissue and represents approximately 20% of non-collagenous proteins.
   1.2. ELSA-OSTEO is a solid-phase immunoradiometric assay. Two monoclonal antibodies were prepared against sterically remote sites. The first is coated on the ELSA solid phase, the second is radiolabeled with iodine 125.
   1.3. Osteocalcin molecules present in the standards or samples are sandwiched between the two antibodies. Excess unbound tracer is removed during a wash procedure, and the ELSA retains only the absorbed antibody/antigen/tracer antibody combination. The amount of radioactivity bound to the ELSA is proportional to the amount of osteocalcin present at the beginning of the assay.

2. Specimen:
   2.1. The assay is performed on serum or plasma. Heparin or EDTA plasma samples may be used. Citrate plasma samples should not be used.
   2.2. Hemolyzed or hyperlipemic samples should not be used.
   2.3. Samples must be frozen at -20°C if not analyzed immediately.

3. Reagents:
   3.1. The following are supplied in a ELSA-OSTEO kit.
   3.2. ELSA Tubes
      3.2.1. 48 tubes contain monoclonal anti-human osteocalcin antibody coated on ELSA. The ELSA is fixed to the bottom of the tubes.
      3.2.2. Tubes are stored at 2-8°C until the expiration date. Tubes removed from packages must be stored in the zip lock bag provided in the kit.
   3.3. ANTI-OSTEO I^{125}
      3.3.1. The 16 mL vial contains ≤ 8 μCi of I^{125} monoclonal antihuman osteocalcin antibody, buffer, animal proteins, sodium azide, red dye, non-immunized mice immunoglobulins.
      3.3.2. Store at 2-8°C until expiration date.
   3.4. Standard 0
      3.4.1. Lyophilized buffer and animal proteins.
      3.4.2. Reconstitute the vial's contents by volumetrically adding 4 mL of deionized water.
      3.4.3. Store at 2-8°C until expiration date. After reconstitution store at -20°C for one month.
   3.5. Standards 1 - 4
      3.5.1. Lyophilized buffer, animal proteins, human osteocalcin.
      3.5.2. Reconstitute the vials' contents by volumetrically adding 0.5 mL of deionized water.
      3.5.3. Store at 2-8°C until expiration date. After reconstitution store at -20°C for one month.
   3.6. Control
      3.6.1. Lyophilized buffer, animal proteins, human osteocalcin.
      3.6.2. Reconstitute the vials contents by volumetrically adding 0.5 mL of deionized water.
      3.6.3. Store at 2-8°C until expiration date. After reconstitution store at -20°C for one month.
   3.7. TWEEN 20:
      3.7.1. Dilute 3 mL of TWEEN 20 in one liter of deionized water.
      3.7.2. Store at 2-8°C until expiration date. After dilution store in a capped container for a maximum of 15 days.

4. Equipment and Supplies:
   4.1. MLA pipet or equivalent pipet capable of accurately dispensing 50 μl.
   4.2. Eppendorf repeating pipet.
   4.3. Eppendorf Combitip. Two sizes needed.
      4.3.1. A tip which can accurately dispense 300 μl.
      4.3.2. A tip which can accurately dispense 3 ml.
   4.4. Vortex
   4.5. Rotating shaker capable of rotating at 175 rotations/min.
   4.6. Foam decanter.
   4.7. Decanting blotters.
   4.8. Gamma Counter with Iso-data software.

5. Calibration:
   5.1. Five calibrating standards are included in the kit.

5.2. Each standard must be volumetrically reconstituted with deionized H_20. Let reconstituted standards sit for 15 minutes before use.
5.3. Reconstituted standards are stable for one month at -20°C.
5.4. Avoid repeated freeze thaw.
5.5. The standards are set up in triplicate.
5.6. Any triplicate that has a CV over 10% must be reviewed by a supervisor to evaluate the effect on the curve.
5.7. The actual dose result and the calculated dose result must not differ by more than 15% for each standard.

6. Quality Control:
6.1. One quality control sample is supplied..
6.2. The control is reconstituted with 0.5 ml of deionized water. Let reconstituted control sit for 15 minutes before use.
6.3. Reconstituted control is stable for one month at -20°C.
6.4. Avoid repeated freeze-thaw.
6.5. Control is set up in duplicate at the beginning and end of each run.
6.6. If the duplicate control tubes have a CV of over 10% the run must be repeated.
6.7. If the control is outside its 2 SD range the run must be repeated.

7. Assay Procedure:
7.1. All reagents must be brought to room temperature (18 -25°C) at least 30 minutes before their use. Standards and control must be reconstituted 15 minutes before use.
7.2. Label appropriate ELSA tubes in triplicate for the standards and in duplicate for the controls and samples.
7.3. Use a MLA pipet to dispense 50μl of standards, controls, or samples into the corresponding - labeled ELSA tube.
7.4. Add 300 μl of I^{125} anti-human osteocalcin to each ELSA tube with an Eppendorf pipet.
7.5. Vortex each tube
7.6. Parafilm and incubate for 2 hours ± 5 minutes at room temperature (18-25°C) on a rotating shaker at a 175 rotations/minute.
7.7. Use Eppendorf to dispense 3 ml of diluted Tween 20 wash solution into each ELSA tube.
7.8. Place tubes into a foam decanter and decant liquid.
7.9. Repeat wash process twice for a total of 3 washes.
7.10. Blot tubes by inverting and tapping tubes on a blotting pad.
7.11. Measure the remaining radioactivity bound to the ESA on a gamma counter for one minute per tube.

8. Calculations:
8.1. Use Iso data reduction software to do the following:
8.1.1. Calculate B/B0 for each tube using the 0 standard as B0.
8.1.2. Calculate the mean of the triplicate results for each standard.
8.1.3. Use a Linear v. Linear graph to plot dose response v. mean B/B0.
8.1.4. Draw a weighted -linear line through the mean of each standard.
8.1.5. Calculate the dose response of each duplicate control and sample from the curve drawn.

9. Reporting Results:
9.1. Results are reported to one decimal point in ng/mL.
9.2. Duplicate sample tubes with CV > 10% must be repeated.

9.3. Results are reported from 0.4 ng/mL to value of high standard.
9.4. Reference Range:

The chart below shows the distribution of values obtained in presumably normal adult subjects.

	Age (years)	Number of cases	Mean ng/ml	Median ng/ml	Extreme values ng/ml
Males	20 - 30	48	23.8	22.0	11.3 - 37.0
	31 - 40	51	21.5	19.4	10.7 - 34.1
	41 - 50	49	20.3	19.9	5.2 - 34.5
	51 - 60	91	18.7	18.6	6.3 - 30.7
	61 - 70	60	19.1	19.2	8.8 - 29.7
Females	20 - 30	70	21.8	21.1	8.8 - 39.4
	31 - 40	87	17.1	16.2	7.7 - 31.9
	41 - 50	74	15.7	15.0	8.0 - 36.0
	51 - 60	85	24.4	22.6	8.0 - 50.5
	61 - 70	32	24.4	24.3	12.9 - 55.9

SIGNATURES

Procedure LAB #4032

Originator(s): Susan C. Baker Date: 3-15-97

_____ Date: _____

_____ Date: _____

_____ Date: _____

Approved By: (must be signed by either the President, Vice President of Laboratory Operations, Vice President of Client Services, Vice President of Clinical Biostatistics and Research Data Systems, or Manager of Quality Control Research and Development.)

Approval: Judy Miller Date: 3/15/97

Title: _____

Yearly Review:

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

College of American Pathologists

Accredited Laboratory

The College of American Pathologists

certifies that the laboratory named below

Medical Research Laboratories
Evan A. Stein, MD, PhD

Laboratory Number: 31719-01

has met all applicable standards for accreditation and is hereby fully accredited by the College of American Pathologists Laboratory Accreditation Program. Reinspection should occur within 30 days of February 15, 1999 to maintain accreditation.

Accreditation does not automatically survive a change in director, ownership, or location and assumes that all interim requirements are met.

William B. Hamlin, M.D.
Commissioner

Raymond C. Zarrow MD
President

CENTERS FOR DISEASE CONTROL AND PREVENTION
NATIONAL HEART, LUNG, and BLOOD INSTITUTE
LIPID STANDARDIZATION PROGRAM

This certifies that

Medical Research Laboratories

Highland Heights, Kentucky

under the direction of Evan A. Stein, M.D., Ph.D.
has successfully met the criteria of precision and accuracy for the measurement of
Hitachi 747

Total Cholesterol	QTR I	○	○	○
HDL - Cholesterol	QTR I	○	○	○
Triglyceride	QTR I	○	○	○

as Specified for Standardization by the Centers for Disease Control-National Heart, Lung and Blood Institute Lipid Standardization Program

Gerald R Cooper
Medical Director

1997

Gary L. Myers
Scientific Director